EXHIBIT A

Docket No.: 066654-0622 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Lipton, Stuart A., et al.	Customer No.: 41552
Appl. No.	:	09/876,187	Confirmation No.: 5845
Filed	:	June 05, 2001	
Title	:	METHODS OF DIFFERENTIATING AND PROTECTING CELLS BY MODULATING THE P38/MEF2 PATHWAY	
Grp./A.U.	:	1632	
Examiner:	:	Anne Marie Falk	

Declaration Pursuant to 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Stuart A. Lipton, declare as follows:

1) I am the Stuart A. Lipton who is named as a co-inventor on the above-identified patent application.

2) I understand that the claims stand rejected, in part, as allegedly lacking enablement.

3) Experimental results are presented herewith that corroborate the enablement of the claimed methods.

4) For generation of neuronal cells from mouse embryonic stem cells, embryonic stem (ES) cells engineered with MEF2CA (constitutively active, MEF2C 1-117/VP16) or MEF2DN (dominant negative, MEF2C 1-105 flag) and an enhanced green fluorescent protein (EGFP) reporter gene under the control of a Nestin/tk promoter were used. The undifferentiated D3 ES cell line was cultured on gelatin-coated plates deprived of feeder cells. Cells were trypsinized into single cells and transfected with pNestin/TKMEF2CA, pNestin/TK-MEF2DN,

or pNestin/TKEGFP by electroporation (0.25 kV, 500mF). Cells were resuspended in a complete medium and placed in 100-mm bacterial grade dishes to allow the cells to aggregate in suspension in the absence of leukemia inhibitory factor (LIF). This is defined as day 0 of embryoid body (EB) formation. On day four, all-trans retinoic acid (RA, 5 μM) was added and left for the last four days. On day six, in addition to all-trans RA, 200 μg/mL Geneticin (G418) was added to the EBs, and EBs were maintained for two more days. Geneticin is used to eliminate non-transfected cells from the mixed population of differentiated ES cell progeny. The transfected clones were selected by growth in the presence of G418 (200mg/ml). The selected clones were then screened by the expression of EGFP, and the clones showing the highest levels of expression were chosen for further expansion and differentiation experiments. Wild-type D3 and EGFP transformed ES cells were used as controls. After eight days, the embryoid bodies were dissociated and plated on tissue culture plates for expansion and subcloning in serum-free medium containing bFGF and EGF.

5) For lineage selection of EGFP-positive neuronal progenitors, embryoid bodies expressing EGFP began to appear within less than one day after addition of RA and G418. These EGFP-positive primary EBs contained neural stem cells (NSCs), including neuronal and glial progenitors, in different states of differentiation. EBs showing the highest levels of EGFP expression were chosen for further subcloning. Using EGFP-positive EBs as the source, MEF2CA-ES-derived or control EGFP-ES-derived EBs were mechanically dissociated and plated in a defined serum-free medium in the presence of a mitogenic factor (EGF or bFGF). Neural stem cells began to proliferate after about 24 hours in culture and formed small clonal clusters of cells by two days. The clusters continued to grow in size, and by day 3–5 the majority of the clusters detached from the substrate and floated in suspension. By day seven, the clonal clusters, called neurospheres, typically measured 100-200 μm in diameter, were composed of approximately 10,000 cells and were ready to be passaged. Neurospheres were mechanically dissociated into a single cell suspension and replated under the same conditions as the primary culture. All these mitotic cells were green, indicating they were proliferating nestin-positive neural stem cells, a conclusion supported by staining experiments in which an antibody was used that recognizes the intermediate filament protein nestin. In addition to expression of EGFP, the

mitotic MEF2CA-ES-derived neuronal progenitors also expressed a constitutively active form of MEF2. In contrast, MEF2 expression could not be detected in control progenitors.

6) Immunocytochemistry studies were performed two days after plating in the culture condition with FGF2 and EGF. The proneuronal basic helix-loop-helix (bHLH) transcription factor MASH1 is a positive regulator of neurogenesis. The proneuronal bHLH transcription factor MASH1 was upregulated in MEF2CA-ES-derived neuronal progenitor cells. Compared to control EGFP-ES-derived progenitor cells, a high percentage of NCAM-positive cells were also found in MEF2CA-ES-derived neuronal progenitor cells. Similarly, increased expression of another neuronally restricted protein, doublecortin (Dcx), was also observed in MEF2CA-ES derived neuronal progenitor cells.

7) Mice were injected with a small number (50,000 cells) of control or MEF2CA-ES-derived neuronal progenitors labeled with bromodeoxyuridine (BrdU) along the anterior-posterior axis of the ipsilateral cortex one day after a 60-minute transient middle cerebral artery occlusion (tMCAO) (Exhibit 1, Figure 5-1). Labeling with BrdU allows visualization of viable grafted cells that are capable of dividing. At 1 day post-grafting, the cells were uniformly and heavily labeled and clustered at the center of the injection site. Four weeks later, brain sections were stained with anti-green fluorescent protein antibody to identify the transplanted cells. Large numbers of the transplanted MEF2CA-ES-derived neuronal progenitor cells survived around the injection site and expressed the immature neuronal marker β-tubulin II (TUJ1) (Exhibit 2, Figure 5-2). A number of MEF2CA-ES-derived neuronal progenitor cells migrated out from the injection site into the ischemic core. These results show that MEF2CA-ES-derived neuronal progenitors can survive in ischemic mouse brain.

8) At 1 day post-transplantation, all of the grafted cells expressed EGFP. Then EGFP was down regulated in the majority of the cells in the days following engrafting. This down regulation of EGFP label occurred because as the MEF2CA-ES-derived neuronal precursor cells matured into neurons, less nestin was expressed and hence the nestin/tk promoter which drives EGFP expression was gradually turned off. Therefore, to better visualize the pattern and fate of engrafted cells *in vivo*, MEF2CA neuronal progenitor cells were labeled with

cell tracker green (CTG) before transplantation. Cell Tracker Green can freely pass through cell membranes and, once inside a cell, undergoes a series of specific reactions, producing a cell-impermeant fluorescent dye that is susceptible to aldehyde fixatives. This probe is retained in living cells and is not transferred among adjacent cells in a population. Eight weeks later, mice brains were perfused and sectioned. Immunofluorescence labeling for any remaining EGFP using anti-EGFP antibody along with the CTG label to identify the transplanted cells was combined with labeling for NeuN in order to identify neurons (Exhibit 3, Figure 5-3). Note the NeuN immunoreactive neurons (red, open arrow) forming the granule cell layer of the dentate gyrus. The yellow color indicates grafted cells adjacent to the lesion site doubled-labeled for NeuN (red) and GFP (green). Cells did not survive when transplanted into the lesion cavity but did survive when transplanted adjacent to the lesion cavity. Representative images show differentiation of transplanted MEF2CA-ES-derived neuronal progenitor cells (green) into neurons (NeuN in red) at low (Exhibit 3, Figure 5-3), medium and high magnification (Exhibit 4, Figure 5-4). Low magnification shows the distribution of green transplanted cells (red arrows) within the tissue, appearing yellow (black arrows) when stained for NeuN. Medium magnification shows the migration of grafted cells into the ischemic area. The higher magnification insert confirmed the co-labeling of both markers in the same cell. Virtually all of transplanted cells migrating from the graft were neuronal, not astrocytic. These results show the distribution and differentiation of transplanted MEF2CA-neuronal progenitor cells eight weeks post-implantation in adult mouse ischemic brain. None of the injected cells formed teratomas.

9) Following grafting of these genetically labeled, EGFP/CTG-positive, MEF2CA-engineered neuronal precursors into the mouse ischemic brain, functional characterization of the engrafted cells was performed by electrophysiology using patch electrodes in current-clamp and voltage-clamp modes. EGFP/CTG-positive cells were detected with neuronal morphology in acute hippocampal slices eight weeks following transplantation. Action potentials were recorded, which were blocked by the addition of 1 μM tetrodotoxin (Exhibit 5, Figure 5-5A). Miniature excitatory postsynaptic currents (mESPCs), recorded under voltage clamp, indicated that the engrafted cells had made synaptic connections (Exhibit 5, Figure 5-5B). Fluorescence and IR-DIC imaging of live slices allowed electrophysiological recordings from EGFP/CTG-

positive cells. No gross morphological malformations or damage to the hippocampus owing to the injection procedure was seen in any of the grafted animals. Data were collected from 20 cells recorded from 20 slices that were prepared from a total of 10 animals. These results describe the electrical properties of transplanted MEF2CA-ES-derived cells.

10) In conclusion, these results show that transplanted MEF2CA neuronal stem cells survive, migrate and differentiate into neurons in the ischemic mouse cerebral cortex.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

7/12/07
Date

Stuart A. Lipton, M.D., Ph.D.

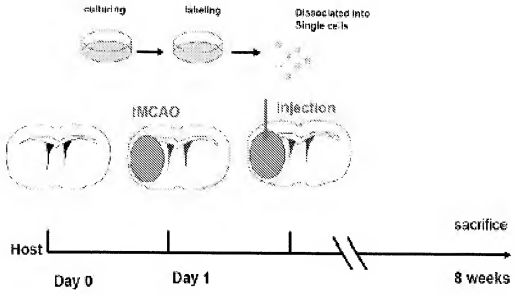

Figure 5-1: Time line for stem cell transplantation in a mouse model of stroke. The middle cerebral artery was occluded (MCAO) for 1 hour, followed by 24 hours reperfusion. The next day, MEF2CA-ES-derived cells or control EGFP-only-ES-derived cells were injected into the lesion site.

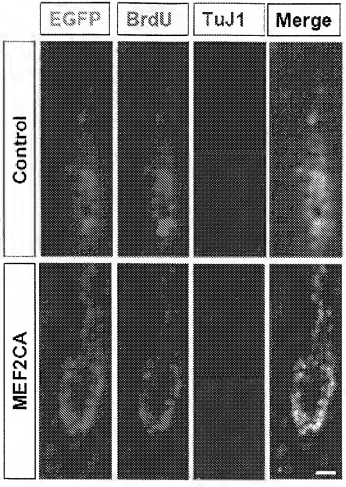

Figure 5-2. Neuronal differentiation of grafted MEF2CA-NSCs in acute mouse stroke model one month after transplantation.
Data showing distribution of transplanted cells one-month post-grafting. The vast majority of the transplanted MEF2CA-ES-derived progenitor cells expressed the early neuronal marker TuJ1. In contrast, virtually none of the EGFP-only cells expressed TuJ1. Scale bar is equivalent to 25 μm for all panels.

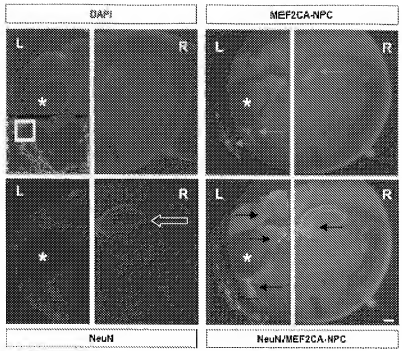

Figure 5-3. Distribution and differentiation of transplanted MEF2CA-NPCs 8 weeks post-implantation in adult mouse ischemic brain.
Representative images showing differentiation of transplanted MEF2CA-ES derived neuronal progenitor cells (green) into neurons (NeuN, red) at low power magnification (2x). Transplanted cells that differentiated into neurons were dual-labeled and thus yellow and are located outside of the ischemic core (*). DAPI (blue) labeled cell nuclei. Scale bar is equivalent to 200 μm for all panels.

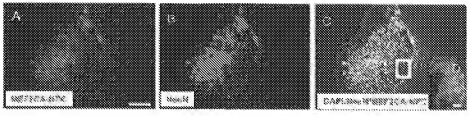

Figure 5-4. Distribution and differentiation of transplanted MEF2CA-NPCs 8 weeks post-implantation in adult mouse ischemic brain at higher magnification.
(A-C) Medium magnification (10x) revealed widespread migration of engrafted MEF2CA-ES-derived neuronal progenitor cells into host brain parenchyma. *(D)* Higher magnification (box in *A*) confirmed co-labeling of transplanted neuronal progenitor cells (green) and NeuN (red), identifying the cells as engrafted neurons (yellow). Scale bar is equivalent to 200 μm for A, B, C (Inset, 15 μm).

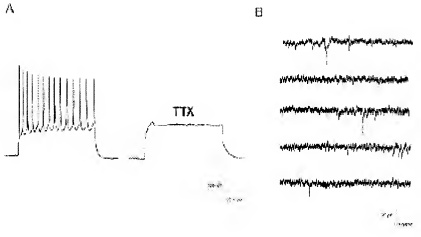

Figure 5-5. Functional characterization of a representative engrafted MEF2CA-NPCs by electrophysiology. *(A)* Hippocampal slice recording under current clamp with a patch electrode. Action potentials were present (left) and blocked by addition of tetrodotoxin (TTX). *(B)* The presence of miniature excitatory postsynaptic currents (mEPSCs) indicate that the engrafted cells had formed functional synapses.

EXHIBIT B

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Neurobiology

Antiapoptotic role of the p38 mitogen-activated protein kinase-myocyte enhancer factor 2 transcription factor pathway during neuronal differentiation

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Myocyte enhancer factor 2 (MEF2) is in the MADS (MCM1agamous-deficiens-serum response factor) family of transcription factors. Although MEF2 is known as a myogenie factor, the expression pattern of the MEF2 family of genes (MEF2A-D) in developing brain also suggests a role in neurogenesis. Here we show that transfeetion with MEF2C, the predominant form in mammalian cerebral cortex, induces a mixed neuronal/myogenic phenotype in undifferentiated P19 precursor cells.

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